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Full Assessment of C_4 Photosynthesis in *Blepharis attenuata* Napper (Acanthaceae) from Jordan: Evidence from Leaf Anatomy and Key C_4 Photosynthetic Enzymes

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Abstract: *Blepharis* constitutes an important element of the desert vegetation in the Middle East, inhabiting extreme desert habitats of the Saharo-Arabian and Sudanian regions. It is the only genus in the Acanthaceae that includes C_4 species; however, comprehensive studies for these species are generally absent. This study presents a thorough assessment of C_4 biochemical subtype in *B. attenuata* Napper through investigating leaf ultrastructural features and activities of C_4 acid decarboxylating enzymes. Leaf anatomy of *B. attenuata* revealed a typical atriplicoid Kranz type, with prominent chlorenchymatous bundle sheath and mesophyll tissues forming concentric rings around leaf veins. Transmission electron microscopy showed characteristic features of NADP-malic enzyme subtype, with bundle sheath chloroplasts having grana with reduced thylakoid appression compared to mesophyll counterparts. Immunolocalizations of key C_4 pathway enzymes showed a strict compartmentalization patterns between bundle sheath and mesophyll, with Rubisco localized in the bundle sheath and both PEP-carboxylase and pyruvate, orthophosphate dikinase were confined to the mesophyll. Measurements of enzyme activities showed that NADP-malic enzyme demonstrated the highest activity, suggesting that *B. attenuata* would best be classified as a NADP-malic enzyme subtype C_4 species. This study reveals that *Blepharis* encompasses at least two biochemical subtypes of C_4 photosynthesis within the genus.

Key words: C_4 photosynthesis, Kranz anatomy, *Blepharis*, Jordan, immunolocalization, NADP-ME subtype

INTRODUCTION

C_4 photosynthesis is an evolutionary response to enzymatic inefficiency in carbon fixation under high temperatures, light intensities, drought and saline stresses (Sage, 2001, 2004; Al-Shoaibi, 2008). The main photosynthetic enzyme Ribulose-1,5-bisphosphate caboxylase/oxygenase (Rubisco) is a "dual" enzyme and under stressful conditions, its oxygenase activity leading to photorespiration is enhanced relative to its carboxylase activity. C_4 plants overcome this limitation by concentrating CO_2 around Rubisco so that its efficiency in carbon fixation is greatly improved, whereas photorespiration is depressed (Kanai and Edwards, 1999). This is commonly achieved in C_4 plants through metabolic cooperation of two distinctive and specialized chlorenchyma tissues, mesophyll (M) and bundle sheath (BS), which are proximally arranged for rapid translocation of metabolites and termed Kranz type anatomy (Dengler and Nelson, 1999; Dengler and Taylor, 2000; Muhaidat *et al*., 2007; Edwards and Voznesenskya, 2011). In C_4 photosynthesis, CO_2 is initially fixed as C_4 acids by phosphoenolpyruvate carboxylase (PEPC) in M cells. Then, C_4 products are shuttled into the BS cells, and decarboxylated (Kanai and Edwards, 1999). CO_2 released is re-fixed by Rubisco, while C_3 acids return into M cells to perpetuate the C_4 cycle. The C_4 pathway is a sophisticated mechanism allowing for CO_2 capture at considerably low CO_2 levels in the leaf and results in improved carbon uptake, rapid growth and efficient use of water and nitrogen under particular environmental conditions (Sage and Pearcy, 2000). For this reason, C_4 plants flourish chiefly in open and hot habitats of the tropics and subtropics with sparse rainfall and long, dry seasons (Sage *et al*., 1999a; Sikolia *et al*., 2009; Salehi *et al*., 2012).

Phylogenetic analyses in the angiosperms have shown that C_4 photosynthesis has evolved independently on numerous occasions in 19 families, producing more than 60 C_4 lineages with a great diversity in Kranz

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anatomy and biochemistry (Sage *et al*., 2011; Gowik and Westhoff, 2011). Three biochemical subtypes of C_4 photosynthesis exist amongst these lineages relating to the predominant activity of the C_4 acid decarboxylating enzyme, including NADP- and NAD- malic enzyme (NADP-ME, NAD-ME) and PEP-carboxykinase (PCK) (Sage *et al*., 2011; Edwards and Voznesenskya, 2011). All three subtypes are found in the C_4 grass lineages, whereas only two principal subtypes (NAD-ME and NADP-ME) occur in the C_4 eudicot lineages (Muhaidat *et al*., 2007). To date, only three C_4 eudicot lineages are known to include both NADP-ME and NAD-ME subtypes. These are *Sesuvium/Trianthema /Zaleya* (Aizoaceae), *Chrysanthellum/Isostigma* (Asteraceae) and *Portulaca* (Portulacaceae) (Muhaidat *et al*., 2007; Voznesenskaya *et al*., 2010; Sage *et al*., 2011). However, surveys are incomplete for a number of C_4 eudicot lineages and the spectrum of C_4 diversity within these lineages remains enigmatic.

Blepharis (Acanthaceae), a pantropical genus of arid and semi-arid regions of the old world, is one of the least explored C_4 eudicot lineages. It is the only genus in the Acanthaceae containing C_4 species (Sage, 2004). The genus is large, consisting of 129 species of annual or perennial herbs and shrubs and is most diverse in tropical and Southern Africa (Vollesen, 2000, 2002). In the Middle East, *Blepharis* constitutes an important element of the desert vegetation inhabiting extreme desert habitats of the Saharo-Arabian and Sudanian regions receiving scarce rain in winter and very high temperatures and intense light during summer (Gutterman, 2002). Two species of the genus have been widely accepted to occur in these regions and were generally recognized as distinct: *B. ciliaris* [L.] B. L. Burtt and *B. attenuata* Napper (Feinbrun-Dothan, 1978; Gutterman, 2002). However, this opinion has not been advocated by Vollesen (2000), who argued in his taxonomic revision of the genus that *B. attenuata* is the only *Blepharis* member that migrated north and colonized desert areas of Egypt, West bank (Israel/Palestine) and Jordan. Within Jordan, *Blepharis* inhabits the drier parts of the lower and upper Jordan valley, the Dead Sea, Araba, Al-Yotm and Rum regions (Feinbrun-Dothan, 1978; Gutterman, 2002). Here, the genus can be readily distinguished on stony hilltops, arid slopes and in wadi beds on rocky grounds, runnels and crevices all the year-round (Fig. 1).

Screening for C_4 photosynthesis in *Blepharis* has largely been based on measuring carbon isotope ratios ($\delta^{13}C$) using herbarium specimens. Using this method, 20 *Blepharis* species belonging to section *Acanthodium* were found to have the C_4-type isotope signature (Sage *et al*., 1999b, 2011). This information, however, is limited by the paucity of information about leaf anatomical, biochemical and physiological features of C_4 photosynthesis in *Blepharis*. To the best of our knowledge, only two C_4 *Blepharis* species have been subtyped based on biochemical assays of the C_4 decarboxylating enzymes and/or ultrastructural analyses (Muhaidat *et al*., 2007; Akhani *et al*., 2008). Muhaidat *et al*. (2007) recorded high activities of NADP-ME in leaves of *B. attenuata* (presumably misidentified as *B. ciliaris*) from Jordan suggesting NADP-ME C_4 subtype. Akhani *et al*. (2008) presented data for *B. ciliaris* from Iran based on immunoblotting of the C_4 decarboxylases (showing a clear-cut immunoreactive band for NAD-ME C_4 isoform) and BS cell ultrastructure (grana chloroplasts and numerous well-developed mitochondria), indicative of NAD-ME subtype. However, it would be instructive to verify names of Jordanian C_4 *Blepharis* species and to further clarify its C_4 biochemical subtype to come to definitive conclusions.

The present study aimed first at confirming identity of Jordanian *Blepharis* species using characters relating to general growth habit and inflorescence. The second aim of this study was to reassess the C_4 subtype of Jordanian *Blepharis* species, with particular emphasis on elucidating its Kranz features and measuring activities of the C_4 decarboxylating enzymes. To provide a full assessment of C_4 photosynthesis in Jordanian *Blepharis*, the intercellular accumulation patterns of key C_4 enzymes were also examined. This study is the first to provide conclusive lines of evidence from leaf anatomy in support of previously published enzyme activity data on an incorrectly identified *B. attenuata*.

MATERIALS AND METHODS

Plant materials and species identification: Mature capsules of *Blepharis* were collected during October, 2010 from separate plants growing in the Dead Sea Valley (31°74′880"N; 35°59′378"E, altitude - 381 m below sea level) and lower slopes of Kufranjah, Ajlun (32°24′770"N; 35°61′646"E, altitude - 180 m below sea level) (Fig. 1). Although previously misidentified as *B. ciliaris* (Muhaidat *et al*., 2007), the species was identified as *B. Attenuata* using characters relating to general growth habit, leaves, bracts and flowers and in reference to the floristic work by Vollesen (2000). Voucher specimens of the species from both sampling sites were deposited in the Herbarium at the Department of Biological Sciences, Yarmouk University.

Plant growth conditions: Collected capsules were split into halves and seeds were sown in 500 mL plastic pots

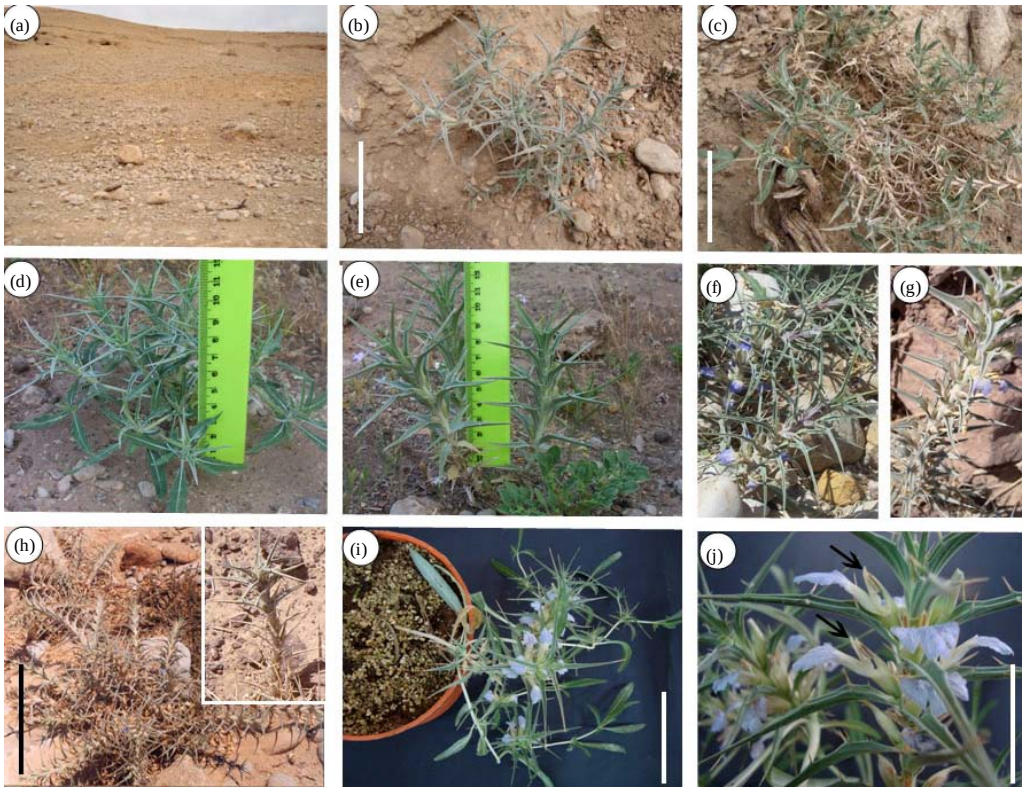

Fig. 1(a-j): *Blepharis attenuata*. (a) Lower arid slope of Kufranjah region (sampling site), Northern Jordan. (b-h) Growth habits (vegetative and reproductive) of the species in areas of collection. (b) Natural habit in Kufranjah. (c-h) Natural habits in the Dead Sea region. Note the marked spiny habit in b, e, f, g and h. Note also the dead spikes with mature capsules from past years on a plant of recent growth in c. (h, insertion) green spike overtopping old capsule-containing spike. (i) Growth habit of a greenhouse-cultivated plant with spike-type inflorescence. J is a close-up of I. Appendaged stamens (unlabeled arrowheads). Scale bars: (b, c, h and i) 5 cm, (h) 10 cm and (j) 2 cm

filled with a soil mixture of 2:1:1 top (soil:sand:commercial potting soil) (Biolan Oy, Kauttua, Finland). They were grown in a naturally-illuminated greenhouse and watered by absorption from the bottom every other day. Fully-grown plants were transplanted into 4 L plastic pots filled with the same soil mix and maintained under natural light with midday photosynthetic photon fluxes of ~900-1200 μmol m^{-2} sec^{-1} (measured using a LI-250A light meter, LI-COR, USA), 14/10 h light/dark photoperiod, ~30-35/20-25°C day/night temperature regime and a relative humidity of 30-40% (measured using a Thermo Hygrometer, Taylor Sybron, Mexico). Potted plants were watered every other day and fertilized biweekly with Ferti-Green commercial fertilizer (30:10:10%, NPK) (Continental Research Corporation, St. Louis, USA). Recently matured leaves from ~2 months old plants were used in all experiments.

Leaf anatomical study: Tiny pieces of leaf tissues (approx. 1 mm^2) from three plants were prepared for light and electron microscopy following the methods described by Muhaidat *et al*. (2007, 2011) with slight modifications. Leaf tissues sampled from the middle portion of leaf blades were fixed in 1% (v/v) glutaraldehyde in 100 mM (v/v) Na-Cacodylate buffer (pH 7.2), washed in the buffer, and post-fixed in 1% (v/v) OsO_4 in Na-Cacodylate buffer at 4° C. Samples were then dehydrated through an acetone series and embedded in Spurr's resin (Spurr, 1969). Semi-thin sections (0.8 μm) were cut on a Reichert Ultracut ultramicrotome (Reichert-Jung, Vienna, Austria) for bright-field light microscopy, dried onto poly-L-lysine coated slides (100 μg mL^{-1}, MW 560,000) and tissues stained with 1% (w/v) toluidine blue O in 0.1% (w/v) Na_2CO_3. Tissue sections were viewed under bright-field microscopy using Nikon ECLIPSE E400 microscope

(Nikon, Kawasaki, Japan) and digitally imaged using a Nikon digital net camera DN100 (Nikon, Kawasaki, Japan). Ultrathin sections (70-90 nm) were cut using the Ultracut ultramicrotome for transmission electron microscopy and stained with 5% (w/v) uranyl acetate and Reynolds lead citrate (Reynolds, 1963). Sections were examined under a Zeiss EM 10 CR electron microscope (Carl Zeiss NTS GmbH, Jena, Germany) and a series of micrographs for both M and BS cells were taken using a plate and sheet film camera (3 1/4"X4") (Carl Zeiss NTS GmbH, Jena, Germany). The TEM negatives were printed on ILFORD photographic papers (5"X7") and scanned using Epson GT 10000 scanner (Model G650B, Seiko Epson Cord, Japan).

Light microscopic images were analyzed for general patterning of leaf tissues, including arrangement of mesophyll (M) and bundle sheath (BS) cells and organelle distributions. From TEM images, the numbers of chloroplasts, mitochondria and peroxisomes per cell type profile were counted in 18 BS cells and 24 adjacent M cells from three separate leaves at 5,000x magnification. The degree of grana stacking in M and BS chloroplasts was assessed by counting the number of stacked thylakoids in grana from 4 chloroplasts per cell type at 16,000x magnification.

***In situ* C_4 enzyme immunolocalization:** Tissue preparation and immunolocalization of three key enzymes of the C_4 pathway (PEPC, Rubisco and PPdK) in leaves of *B. attenuata* followed exactly the method described by Sudderth *et al.* (2007) and Marshall *et al.* (2007). Antisera used included polyclonal anti-tobacco Rubisco holoenzyme (courtesy of N.G. Dengler, University of Toronto, Canada), anti-maize PEPC (courtesy of J. Berry, State University of New York, USA) and anti-maize PPdK (courtesy of H. Sakakibara, Plant Science Center, Piken, Japan) IgG fractions. Specificity of each antiserum was tested by running control slides incubated with diluting buffer lacking primary antiserum and positive immunolocalization experiments on cross-sections of paraffin-embedded leaf tissues of representative NADP-ME type C_4 (*Zea mays*) and NAD-ME type C_4 (*Amaranthus retroflexus*) in which C_4 enzymes accumulate in specific cells (Edwards *et al.*, 2001). After a standard procedure of leaf tissue fixation, dehydration and immunolabeling, sections were mounted in Canada Balsam (Sigma-Aldrich Co. LLC, St. Louis, MO, USA), examined using bright-field microscopy and digitally imaged.

Photosynthetic enzyme extraction and assay: Enzyme extraction and assay procedures were carried out as previously described by Muhaidat *et al.* (2007). Chlorophyll content was determined following Wintermans and De Mots (1965). Activities of PEPC, NADP-ME, NAD-ME and PCK were measured photometrically at ~30°C and 340 nm using UV-Vis spectrophotometer (PG Instruments Limited, England) (Muhaidat *et al.*, 2007 for detailed description of the extraction and assay procedures).

Statistical analysis: Quantified organelle data were statistically analyzed using SigmaStat software version 3.0 (Systat Inc., San Jose, CA, USA). Mean values were tested using Student's t test. Graphical images label bars with different letters to indicate significant differences at $p \leq 0.05$ ($n \geq 18$).

RESULTS AND DISCUSSION

General morphology of *Blepharis attenuata*: The plant observed and collected from the arid regions around Jordan (Fig. 1a) was identified unequivocally as *Blepharis attenuata*, not *B. ciliaris* as previously reported (Muhaidat *et al.*, 2007). Generally, *Blepharis attenuata* is a spiny perennial C_4 herb, re-sprouting from the apex and bearing few basal leaves at maturity (Fig. 1b-h) (Feinbrun-Dothan, 1978; Vollesen, 2000).

The species is distinctive in having a spike-type inflorescence (of up to 20 cm in greenhouse grown plants; Fig. 1i, j) and flat, lanceolate, leathery leaves with mostly dentate (2-7 teeth per side) margins (Fig. 1d). Closer examination of the leaf arrangement revealed what has previously been designated as a pseudo-whorl phyllotaxy, where "two pairs of leaves at a node are situated just above each other" (Vollesen, 2000). For true whorled phyllotaxy, three or more leaves at a node must be initiated simultaneously (Lee *et al.*, 2009).

On the plant, green spikes with different stages of flower and seed development can be found, in addition to dead spikes from previous years including mature capsules (Fig. 1c, g) (Gutterman, 2002). Green fertile spikes with white, violet, purple and mauve flowers developed distal to older spikes were observed on plants encountered in the field (Fig. 1h, insertion). Bracts are leathery, canaliculate, 3-5 veined with 3-4 pairs of lateral spines, the longest of which is not always longer than the width of the bract as described earlier by Feinbrun-Dothan (1978). The morphology of stamens, specifically shape and length of the appendage characteristic of the anterior pair of stamens, is regarded as one of the most distinguishing characters at the species level in *Blepharis* (Vollesen, 2000). As in many *Blepharis* species, the anterior stamens in *B. attenuata* are laterally flattened, but hairy toward the base, with a flattened and longer appendage than the anther (Fig. 1j, arrowheads).

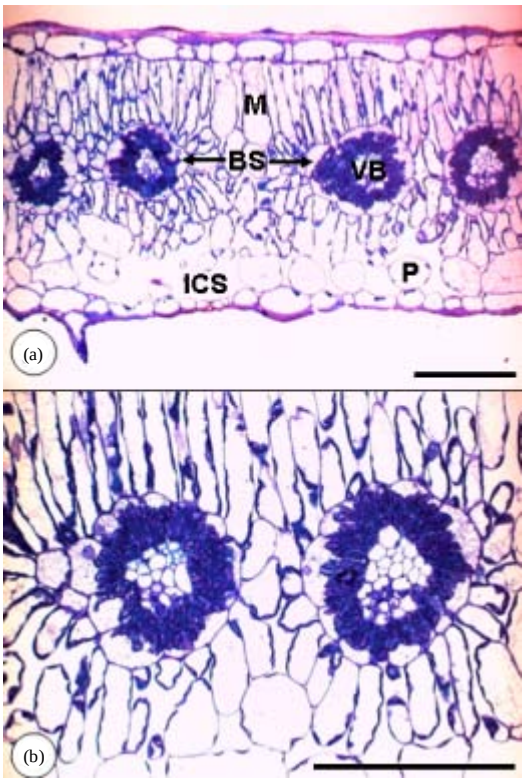

Fig. 2(a-b): Light micrographs of cross sections through leaf blades of *B. attenuata*. (b) higher magnification of (a). The species has the atriplicoid Kranz type, where bundle sheath and mesophyll cells form concentric tissue layers around vascular bundles. Note the centripetal placement of chloroplasts in bundle sheath cells compared to their peripheral distribution in the surrounding mesophyll cells. BS: Bundle sheath, ICS: Intercellular airspace, M: Mesophyll; P: Parenchyma; VB: Vascular Bundle, Scale bars = 100 μm

Kranz anatomy in *Blepharis* leaves: Light microscopic studies showed that leaves of *B. attenuata* have atriplicoid type Kranz anatomy, where prominent BS and M form concentric tissue layers encircling leaf veins (Fig. 2). In *B. attenuata*, individual BS cells are enlarged, cuboidal, thick-walled, and compact containing numerous, large and centripetally-located chloroplasts. The surrounding M tissue consists of thin-walled, radially-arranged cells with fewer chloroplasts located around the periphery of the cell. As observed in other C_4 plants, M cells in *B. attenuata* are exposed to intercellular space, whereas exposure of BS cells to the airspaces is greatly reduced, an anatomical specialization that aids in retaining high CO_2 levels in the BS cells (Dengler and Nelson, 1999; Muhaidat *et al*., 2007). A layer of large and widely-spaced non-chlorenchymatous parenchyma cells (hypodermis) is found adjacent to the abaxial epidermis (Fig. 2). This layer is suggested to act as water storage tissue and support photosynthesis during periods of water stress (Guralnick *et al*., 2002). These particular features are also typical of other atriplicoid Kranz type C_4 eudicots which occurs in about 20 out of 36 C_4 dicot lineages, indicating an extensive evolutionary convergence (Muhaidat *et al*., 2007; McKown and Dengler, 2007; Sage *et al*., 2011).

Transmission electron microscopy confirmed these cellular features, and revealed details of organelle ultrastructure, numbers and distribution (Fig. 3). The BS and M cells exhibit ultrastructural dimorphism typical of C_4 plants, where BS cells possess significantly greater numbers of chloroplasts, mitochondria and peroxisomes per cell profile than their adjacent M cells. Organelle counts show that BS cells contain, on average, 14.6 chloroplasts, 20.3 mitochondria and 4.2 peroxisomes per cell type profile, whereas M cells contain 7.1 chloroplasts, 6.3 mitochondria, and 0.8 peroxisomes (Fig. 4). Dimorphism with respect to sizes of each of these organelles is also apparent when viewed under the same magnification; however, it is most pronounced in case of chloroplasts where the BS chloroplasts are about twice as large as M chloroplasts (Fig. 3a-d). This partitioning of greater numbers and larger organelles into BS cells is an adaptive anatomical feature needed to achieve and process the high levels of CO_2 generated by the C_4 metabolic pump (Dengler and Nelson, 1999).

The chloroplasts of BS and M cells exhibit other ultrastructural features, many of which are typical of NADP-ME subtype C_4 plants. BS chloroplasts show reduction in grana development, whereas M chloroplasts contain well-developed grana (Fig. 3, compare b and c, e and f). Quantitative assessment of the degree of thylakoid stacking shows that BS chloroplasts possess grana of predominantly 2-3 appressed thylakoids (Fig. 5). Grana of higher stacking level are observed in BS chloroplasts but this is not common. By contrast, M chloroplasts have prominent grana stacks with as many as 33 appressed thylakoids per granum. Grana stacks of lower thylakoid stacking level are infrequently observed in the M chloroplasts (Fig. 5).

Generally, the reduced granal development in BS chloroplasts is considered to correlate with deficiency in photosystem (PS) II and linear electron flow activities, resulting in a higher ATP/ NADPH ratio (Edwards and Walker, 1983; Kanai and Edwards, 1999; Takabayashi *et al*., 1969). In the NADP-ME subtype, the export of malate in the C_4 cycle results in both CO_2 and

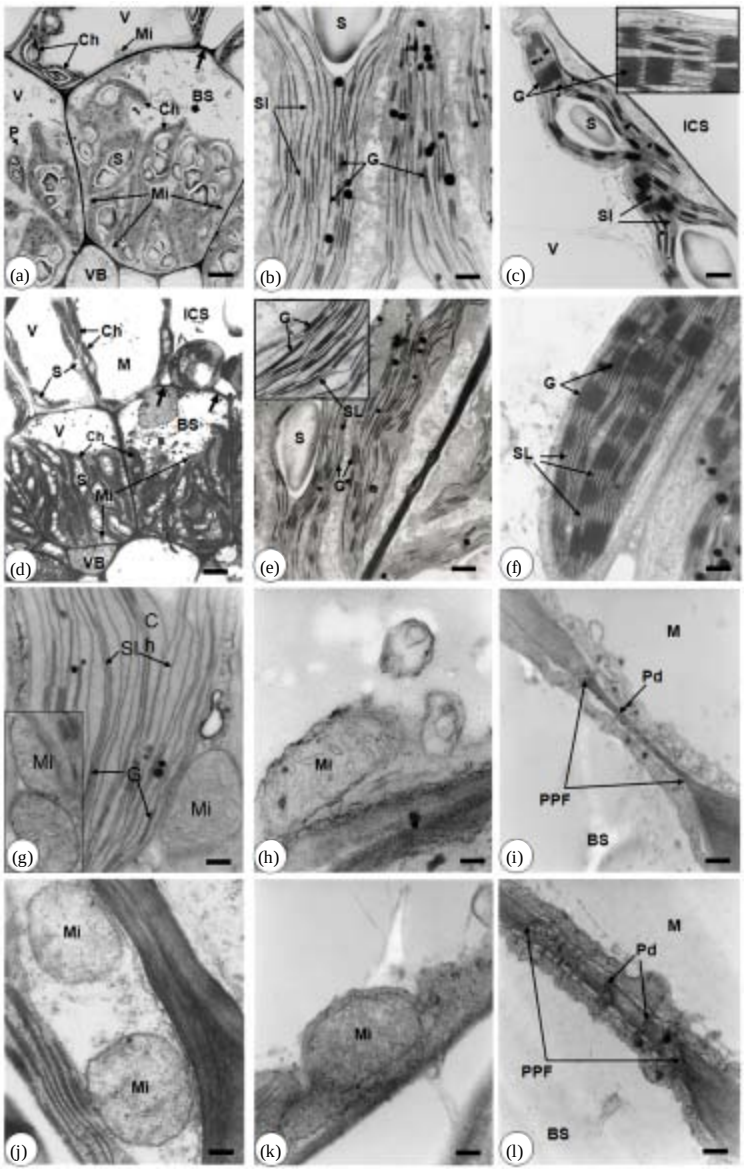

Fig. 3(a-l): Electron micrographs of cross sections through leaf blades of *B. attenuata* illustrating ultrastructural features of bundle sheath and mesophyll cells. (a-c, g-i) *B. attenuata*, Kufranjah region. (d-f, j-l) *B. attenuata*, the Dead Sea region. Note the centripetal placement of organelles in the bundle sheath cells versus their peripheral distribution in the mesophyll cells (a, d). Note also wall thickening at portions of the bundle sheath walls exposed to intercellular space (a, d; arrowheads) and accumulation of starch granules in bundle sheath and mesophyll chloroplasts (a, b, c). (b, e, g) Higher magnification of bundle chloroplasts showing many reduced grana and abundant stroma lamellae. (c, f) Higher magnification of mesophyll chloroplasts showing large grana stacks and short stroma lamellae. Inset in C is a portion of another mesophyll chloroplast. (g, j) Higher magnification of bundle sheath mitochondria and an associated part of bundle sheath chloroplast dominated by stroma lamellae. Inset in G is a higher magnification of mitochondria from another bundle sheath cell. (h, k) Higher magnification of M mitochondria. (i, l) aggregates of plasmodesmata in primary pit field traversing adjoining bundle sheath-mesophyll walls. BS: Bundle sheath, Ch: Chloroplast, G: Granal stack, ICS: Intercellular space, M: Mesophyll, Mi: Mitochondria, Pd: Plasmodesmata, PPF: Primary pit field, S: Starch grain, SL: Stroma lamellae, V: Vacuole, VB: Vascular bundle, Scale bars (a) 3 μm (b) 0.3 μm, (c, e) 0.6 μm (d) 5 μm, (c and e [insets], h, k) 0.1 μm and (f, g, i, j, l) 0.2 μm

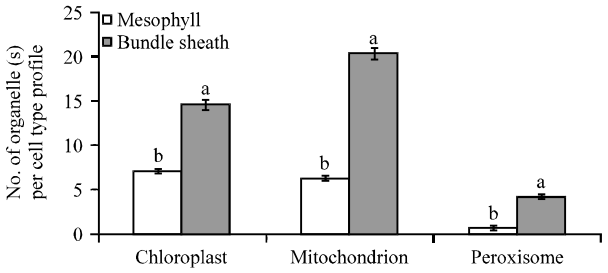

Fig. 4: Numbers of organelles in the mesophyll and bundle sheath cells in leaves of *B. attenuata*. Vertical error bars represent Mean±Standard Error (SE) Different letters indicate a statistically significant difference at $p \leq 0.05$

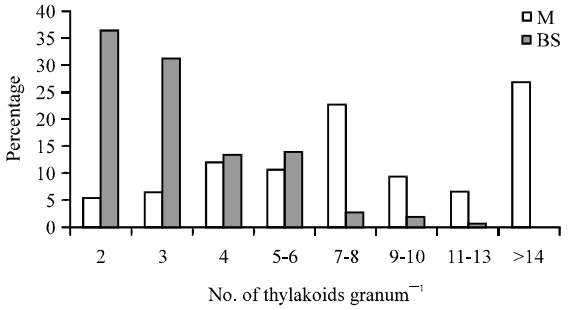

Fig. 5: Relative distribution of thylakoid membranes per granal stack in chloroplasts of the mesophyll and bundle sheath

reducing power to the Calvin cycle through the decarboxylation step in BS cells (Kanai and Edwards, 1999). There is a considerable variation in the level of thylakoid stacking in BS chloroplasts of NADP-ME type C_4 plants. For instance, the BS chloroplasts of *Sorghum bicolor* (monocot) are described to be largely agranal (Edwards and Walker, 1983), whereas BS chloroplasts in a number of other monocot and eudicot species (e.g., *Zea mays*, *Portulaca umbraticola*, *Boerhavia repens* and *Trianthema portulacastrum*) contain a few rudimentary grana (Carolin *et al*., 1978; Voznesenskaya and Gamaley, 1986; Yoshimura *et al*., 2004; Voznesenskaya *et al*., 2010; Muhaidat *et al*., 2011). Other monocot and eudicot NADP-ME C_4 species possess considerable grana stacking in BS chloroplasts (e.g., *Eriachne aristidea*, *Setaria italica*, *Flaveria* spp., *Salsola richteri*, *Haloxylon persicum* and *H. aphyllum*; (Voznesenskaya and Gamaley, 1986; Ketchner and Sayre, 1992; Voznesenskaya *et al*., 1999; Pyankov *et al*., 1999). This apparent reduction in the level of thylakoid appression compared to M counterparts is consistently found in NADP-ME type C_4 plants. The extensive thylakoid stacking in M chloroplasts is associated with enhanced PS-II and non-cyclic electron flow activities generating ATP and NADPH, as NADP-ME type C_4 plants require reducing power for malate biosynthesis (Kanai and Edwards, 1999).

Despite the apparent ultrastructural dimorphism and differences in grana stacking in chloroplast thylakoids, both BS and M chloroplasts are observed containing starch (Fig. 3a-d), indicating that starch synthesis is not restricted to the BS chloroplasts where Rubisco is localized (Edwards *et al*., 2001). Enzymes of the reduction phase of the C_3 cycle accumulate in both M and BS chloroplasts, suggesting that 3-phosphoglycerate exported from BS cells may be engaged for starch synthesis in M chloroplasts (Kanai and Edwards, 1999; Leegood and Walker, 1999). Aggregates of plasmodesmata in primary pit-fields traverse adjoining BS-M walls (Fig. 3i, l), supporting symplastic flux of photosynthetic metabolites between M and BS cells (Dengler and Nelson, 1999).

In *B. attenuata*, the BS mitochondria are numerous, but they exhibit an equally-developed internal membrane system as their counterparts in the adjacent M cells (Fig. 3 compare g and h, j and k). The BS mitochondria are not as large or extensively developed in *B. attenuata* as observed in typical NAD-ME C_4 species (e.g., *Panicum miliaceum*, C_4 species of *Amaranthus*, *Atriplex*, *Calligonum*, *Cleome* and *Suaeda*) (Laetsch, 1968; Gutierrez *et al*., 1974; Hatch *et al*., 1975; Dengler and Nelson, 1999; Yoshimura *et al*., 2004; Edwards and Voznesenskya, 2011). Generally, BS mitochondria in *B. attenuata* are spheroid to partly elongate in cross sections with tubular cristae and interspersed between the BS chloroplasts (Fig. 3g, j). In NAD-ME subtype C_4 plants, mitochondria are the primary CO_2 donating sites to Rubisco and are large, occupying a significant fraction of the BS cell volume compared to BS mitochondria of NADP-ME and PEP-CK C_4 species (Hatch *et al*., 1975; Dengler and Nelson, 1999).

In *B. attenuata*, vacuoles are confined to the centrifugal side of BS cells, whereas they are centralized in M cells. In addition, the walls of BS cells are thicker, and thickening is greatest at sites exposed to intercellular space (Fig. 3a, d; arrowheads). These anatomical specializations, along with the centripetal positioning of organelles, serve as physical barriers to diffusive loss of CO_2, while preserving high CO_2 levels in the BS so that Rubisco becomes CO_2 saturated thereby suppressing rates of photorespiration and operating photosynthesis at a maximal rate (Dengler and Nelson, 1999; Sage, 2004). However, photorespiration, although strongly inhibited, is not entirely eliminated in C_4 species (Ueno *et al*., 2005 and references therein). In the present study, the number

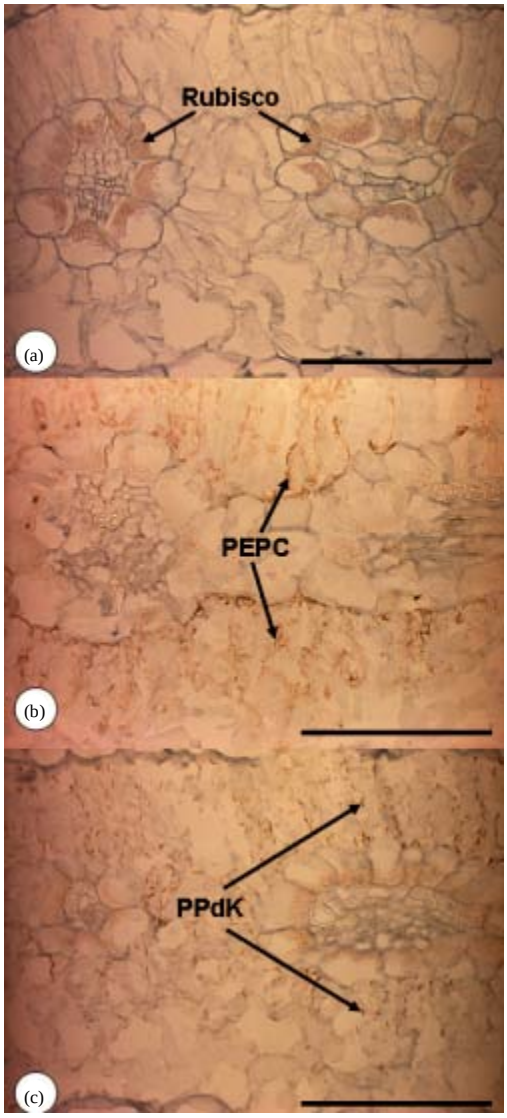

Fig. 6(a-c): Light micrographs of *in situ* immunolocalization of photosynthetic enzymes in cross sections through leaves of *B. attenuata*. Immunolabeling is indicated by the brown precipitate and arrows. (A) Rubisco is strictly localized in bundle sheath cells, (b) PEPC is selectively localized in mesophyll cells, (c) PPdK localization is confined to M cells. Scale bars = 100 μm, Rubisco; Ribulose-1,5-bisphosphate caboxylase/oxygenase, PEPC: Phospho*enol*pyruvate carboxylase, PPdK: Pyruvate, phosphate dikinase

of peroxisomes (where part of the photorespiratory pathway takes place) per BS cell profile was found to be four to five-fold higher than that per M cell profile (Fig. 4) suggesting a CO_2 recapturing mechanism for photorespiration (Bauwe and Kolukisaoglu, 2003; Sage, 2004).

Compartmentalization of photosynthetic enzymes in *Blepharis*: The immunolabeling of photosynthetic enzymes on leaf cross-sections with control C_4 species show specific labeling of each enzyme to a particular tissue (not shown). As well, sections incubated with diluting buffer lacking antiserum show no non-specific binding, confirming the monospecificity of the antibodies used in this study (not shown). Positive immunolabeling is indicated by the brown precipitate. In leaves of *B. attenuata*, the labeling for Rubisco, PEPC and PPdK is strictly compartmentalized between M and BS cells, typical of C_4 plants (Fig. 6). Dense and specific labeling for Rubisco is present in the BS cells, and absent in the M cells (Fig. 6a). In contrast to Rubisco, pronounced labeling for PEPC and PPdK is entirely confined to the M cells adjacent to the BS (Fig. 6b, c). Labeling for PEPC appears distributed throughout the M cells, whereas labeling for PPdK is associated with the M chloroplasts (Fig. 6b, c). No labeling for either PEPC or PPdK in the BS or hypodermal parenchyma tissues is detected. These intercellular compartmentalization patterns coincides with the strict localization of key C_4 enzymes (Edwards *et al*., 2001), supporting fully expressed C_4 photosynthesis in leaves of *B. attenuata*.

C_4 photosynthetic enzyme activity in *Blepharis* leaves: *In vitro* activities of four key photosynthetic enzymes involved in the C_4 pathway are assayed from leaves of *B. attenuata* (Table 1). Leaf extracts from this species contain high PEPC activities, typical of C_4 plants, as PEPC is the primary CO_2-fixing enzyme in C_4 plants (Ashton *et al*., 1990). Among the three C_4-acid decarboxylating enzymes, NADP-ME activity is the highest, being about twelve- to three-fold higher than activities of NAD-ME and PEP-CK, respectively. Differences among these three enzymes are statistically significant ($p<0.001$, Table 1). PEP-CK activity is about three-fold significantly higher than NAD-ME activity. The obtained activity data of decarboxylating enzymes are similar to these published earlier by Muhaidat *et al*. (2007), although *B. attenuata* was misidentified as *B. ciliaris*. The high activity level of NADP-ME supports classification of *B. attenuata* as a NADP-ME C_4 biochemical subtype. The elevated PEP-CK activity in *B. attenuata* is indicative of some involvement of PEP-CK type C_4 cycle in C_4 photosynthesis in the species (Table 1). The presence of PEP-CK in NADP-ME C_4 plants

Table 1: Activities of key photosynthetic enzymes in crude leaf extracts of C_4 *Blepharis attenuata* and comparative C_3 and C_4 species

Species	Photosynthetic type, subtype	Enzyme activity (μmol mg chlorophyll^{-1} h^{-1}) PEPC	NADP-ME	NAD-ME	PEP-CK
Blepharis attenuata	C_4, NADP-ME	1272.2±8.4^a	469.6±5.2^b	40.8±1.9^c	131.1±2.2^d
Control species					
Euphorbia peplus L.	C_3	37.5±3.1	19.9±2.3	11.1±0.9	8.4±0.5
Zea mays L.	C_4, NADP-ME	1246.0±14.1	719.1±3.3	111.6±4.1	152.2±4.1
Amaranthus retroflexus L.	C_4, NAD-ME	1095.1±15.6	84.1±4.6	679.8±10.7	31.0±0.2
Melinis minutiflora Beauv.	C_4, PEP-CK	1030.5±8.6	12.8±0.6	119.3±4.1	572.3±6.0

PEPC: Phosphoenolpyruvate carboxylase; NADP-ME: NADP-malic enzyme, NAD-ME: NAD-malic enzyme, PEP-CK: Phosphoenolpyruvate carboxykinase. Values (Mean±SE) superscripted with different letters indicate significant differences at $p \leq 0.05$ determined by Holm-Sidak test

has previously been reported in some NADP-ME C_4 grasses and eudicots by activity assays (Gutierrez *et al*., 1974; Muhaidat *et al*., 2007), immunoblotting and/or immunolocalization of the C_4 decarboxylases (Wingler *et al*., 1999; Voznesenskaya *et al*., 2006) and *in situ* hybridization of mRNA encoding PEP-CK (Furumoto *et al*., 1999; Marshall *et al*., 2007). The PEP-CK reaction in the BS cytosol is energy-dependent and this energy demand is suggested to be principally met through engagement of NAD-ME type C_4 cycle producing ATP in addition to CO_2 (Kanai and Edwards, 1999; Portis, 2001). The NAD-ME activity reported here, although low, is about 30% of PEP-CK activity, which might be sufficient to drive the PEP-CK decarboxylation reaction. In this study, *B. attenuata* shows a tendency toward some grana development in BS chloroplasts, particularly in the range of 4-6 appressed thylakoids (Fig. 3), reflecting an improved capacity for NADPH production and supports the potential employment of an aspartate-type C_4 cycle (i.e., NAD-ME), the extent of which is proposed to be correlated with the extent of granal development in BS chloroplasts (Voznesenskaya *et al*., 2006). Hence, shuttling of aspartate into BS cells is not accompanied by transfer of reducing power (Edwards and Walker, 1983) and alongside the biochemistry, this ultrastructural adjustment suggests that multiple decarboxylating systems cooperate in CO_2 delivery to Rubisco in leaves of *B. attenuata*.

CONCLUSIONS

The anatomical, biochemical and immunolocalization findings of this study show that *B. attenuata* possesses all features indicative of C_4 photosynthesis. Our study also provides conclusive lines of evidence from leaf ultrastructure and enzyme activity that unequivocally confirm the classification of *B. attenuata* to the NADP-ME subtype. Leaves of *B. attenuata* possess the atriplicoid type Kranz anatomy, with BS chloroplasts of a lower grana development compared to M chloroplasts, and considerably higher activity of NADP-ME than NAD-ME and PCK activities. Our enzyme activity data also indicate a contribution of the PCK type C_4 cycle to carbon assimilation in *B. attenuata*, and may be an adaptive strategy that provides the species with additional energy and competitive edge in extremely hot and arid habitats. This may have some benefit in acclimation to rapid changes in climate. Further, studies (e.g., pulse-chase, immunogold localizations, western and northern blots, *in situ* hybridization) will be required to evaluate the relative functioning of multiple C_4 cycles in *B. attenuata*.

Blepharis attenuata is a characteristic species of the desert in the Sudanian region of Jordan and the west bank (Israel/Palestine), where its distribution is limited by rain and soil humidity. One of the strategies this species has evolved to cope with the extreme desert conditions in the area is C_4 photosynthesis. Findings of this study and those of Akhani *et al*. (2008) support the existence of two C_4 subtypes (NADP-ME subtype *B. attenuata* and NAD-ME subtype *B. ciliaris*) in the *Blepharis* C_4 lineage which is rare in C_4 eudicots. The present study clarified the pattern of C_4 diversity within the genus and provided a firm basis for further exploration of C_4 diversification in the genus throughout its evolutionary history.

To date, *Blepharis* evolution has not yet been extensively sampled in phylogenetic analyses; however, a recent phylogenetic reconstruction of the tribe Acanthaceae recognized *Blepharis* as monophyletic, in spite of the small sampling of *Blepharis* species (McDade *et al*., 2005). Thus, it remains unknown whether C_4 photosynthesis evolved in the genus more than once. Given the existence of two distinct C_4 biochemical subtypes in the *Blepharis* lineage, multiple origins of C_4 photosynthesis in this genus remain a definite possibility. It would be intriguing to address origins and patterns of C_4 evolution in *Blepharis* on a robust phylogeny.

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REFERENCES

Akhani, H., M. Ghasemkhani, S.D. Chuong and G.E. Edwards, 2008. Occurrence and forms of kranz anatomy in photosynthetic organs and characterization of NAD-ME subtype C_4 photosynthesis in *Blepharis ciliaris* (L.) B. L. Burtt (Acanthaceae). J. Exp. Bot., 59: 1755-1765.

Al-Shoaibi, A.A., 2008. Photosynthetic response of elephant grass (*Pennisetum purpureum*) to NaCl salinity. J. Biol. Sci., 8: 610-615.

Ashton, A.R., J.N. Burnell, R.T. Furbank, C.L.D. Jenkins and M.D. Hatch, 1990. Enzymes of C_4 photosynthesis. Methods Plant Biochem., 3: 39-72.

Bauwe, H. and U. Kolukisaoglu, 2003. Genetic manipulation of glycine decarboxylase. J. Exp. Bot., 54: 1523-1535.

Carolin, R.C., S.W.L. Jacobs and M. Vesk, 1978. Kranz cells and mesophyll in the Chenopodiales. Aust. J. Bot., 26: 683-698.

Dengler, N.G. and T. Nelson, 1999. Leaf Structure and Development in C_4 Plants. In: C_4 Plant Biology, Physiological Ecology Series, Sage, R.F. and R.K. Monson (Eds.). Academic Press, San Diego, California, USA, pp: 133-172.

Dengler, N.G. and W.C. Taylor, 2000. Developmental Aspects of C_4 Photosynthesis. In: Photosynthesis: Physiology and Metabolism, Leegood, R.C., T.D. Sharkey and S. von Caemmerer (Eds.). Kluwer Acdemic Publishers, Dordrecht, Netherlands, pp: 471-495.

Edwards, G.E. and D.A. Walker, 1983. C_3, C_4: Mechanisms and Cellular and Environmental Regulation of Photosynthesis. Blackwell Scientific, Oxford.

Edwards, G.E., V.R. Franceschi, M.S.B. Ku, E.V. Voznesenskaya, V.I. Pyankov and C.S. Andreo, 2001. Compartmentation of photosynthesis in cells and tissues of C_4 plants. J. Exp. Bot., 52: 577-590.

Edwards, G.E. and E.V. Voznesenskya, 2011. C_4 Photosynthesis: Kranz Forms and Single-Cell C_4 in Terrestrial Plants. In: C_4 Photosynthesis and Related CO_2 Concentrating Mechanisms, Raghavendra, A.S. and R.F. Sage (Eds.). Springer, Dordrecht, Netherlands, pp: 29-61.

Feinbrun-Dothan, N., 1978. Flora Palaestina: Part 3, Text Ericaceae to Compositae. Israel Academy of Sciences and Humanities, Jerusalem.

Furumoto, T., S. Hata and K. Izui, 1999. cDNA cloning and characterization of maize Phosphoenolpyruvate carboxykinase, a bundle sheath cell-specific enzyme. Plant Mol. Biol., 41: 301-311.

Gowik, U. and P. Westhoff, 2011. The path from C_3 to C_4 photosynthesis. Plant physiol., 155: 56-63.

Guralnick, L.J., G.E. Edwards and M.S.B. Ku, B. Hockema and V.R. Franceschi, 2002. Photosynthetic and anatomical characteristics in the C_4-crassulacean acid metabolism-cycling plant, *Portulaca grandiflora*. Funct. Plant Biol., 29: 763-773.

Gutierrez, M., V.E. Gracen and G.E. Edwards, 1974. Biochemical and cytological relationships in C_4 plants. Planta, 119: 279-300.

Gutterman, Y., 2002. Survival Strategies of Annual Desert Plants. Springer, Berlin, Germany.

Hatch, M.D., T. Kagawa and S. Craig, 1975. Subdivision of C_4 pathway species based on differing C_4 acid decarboxylating systems and ultrastructural features. Aust. J. Plant Physiol., 2: 11-128.

Kanai, R. and G.E. Edwards, 1999. The Biochemistry of C_4 Photosynthesis. In: C_4 Plant Biology, Sage, R.F. and R.K. Monson (Eds.). Academic Press, New York, USA., pp: 49-87.

Ketchner, S.L. and R.T. Sayre, 1992. Characterization of the expression of the photosystem II-oxygen evolving complex in C4 species of *Flaveria*. Plant Physiol., 98: 1154-1162.

Laetsch, W.M., 1968. Chloroplast specialization in dicotyledons possessing the C_4-dicarboxylic acid pathway of photosynthetic CO_2 fixation. Am. J. Bot., 55: 875-883.

Lee, B.H., S.I. Yu and D. Jackson, 2009. Control of plant architecture: The role of phyllotaxy and plastochron. J. Plant Biol., 52: 277-282.

Leegood, R.C. and R.P. Walker, 1999. Regulation of the C_4 Pathway. In: C4 plant biology, Physiological Ecology Series, Sage, R.F. and R.K. Monson (Eds.). Academic Press, San Diego, California, USA., Pp: 89-131.

Marshall, D.M., R. Muhaidat, N.J. Brown, Z. Liu and S. Stanley *et al*., 2007. *Cleome*, a genus closely related to *Arabidopsis*, contains species spanning a developmental progression from C3 to C4 photosynthesis. Plant J., 51: 886-896.

McDade, L.A., T.F. Daniel, C.A. Kiel and K. Vollessen, 2005. Phylogenetic relationships among Acantheae (Acanthaceae): Major lineages present contrasting patterns of molecular evolution and morphological differentiation. Syst. Bot., 30: 834-862.

McKown, A.D. and N.G. Dengler, 2007. Key innovations in the evolution of Kranz anatomy and C_4 vein pattern in *Flaveria* (Asteraceae). Am. J. Bot., 94: 382-399.

Muhaidat, R., R.F. Sage and N.G. Dengler, 2007. Diversity of Kranz anatomy and biochemistry in C_4 eudicots. Am. J. Bot., 94: 362-381.

Muhaidat, R., T.L. Sage, M.W. Fohlich, N.G. Dengler and R.F. Sage, 2011. Characterization of C_3-C_4 intermediate species in the genus *Heliotropium* L. (Boraginaceae): Anatomy, ultrastructure and enzyme activity. Plant Cell Environ., 34: 1723-1736.

Portis, A.R., 2001. Photosynthetic Carbon Metabolism. In: Encyclopedia of Life Sciences, NPG (Ed.). Groves Dictionaries Incorporated, London, ISBN: 9781561592388, pp: 455-457.

Pyankov, V.I., C.C. Black, E.G. Artyusheva, E.V. Vozensenskaya, M.S.B. Ku and G. Edwards, 1999. Features of photosynthesis in *Haloxylon* species of chenopodiaceae that are dominant plants in Central Asia deserts. Plant Cell Physiol., 40: 125-134.

Reynolds, E.S., 1963. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J. Cell Biol., 17: 208-212.

Sage, R.F. and R.W. Pearcy, 2000. The Physiological Ecology of C_4 Photosynthesis. In: Photosynthesis: Physiology and Metabolism, Leegood, R.C., T.D. Sharkey and S. von Caemmerer (Eds.). Kluwer Academic Publishers, Dordrecht, The Netherlands, ISBN: 9780792361435, pp: 497-532.

Sage, R.F., 2001. Environmental and evolutionary preconditions for the origin and diversification of the C_4 photosynthetic syndrome. Plant Biol., 3: 202-213.

Sage, R.F., 2004. The evolution of C_4 photosynthesis. New Phytol., 161: 341-370.

Sage, R.F., D.A. Wedin and M.R. Li, 1999a. The Biogeography of C_4 Photosynthesis: Patterns and Controlling Factors. In: C_4 Plant Biology, Sage, R.F. and R. K. Monson (Eds.). Academic Press, San Diego, California, USA, ISBN: 9780126144406, pp: 313-375.

Sage, R.F., M. Li and R.K. Monson, 1999b. The Taxonomic Distribution of C_4 Photosynthesis. In: C_4 Plant Biology, Sage, R.F. and R.K. Monson (Eds.). Academic Press, San Diego, California, USA., ISBN: 9780126144406, pp: 551- 584.

Sage, R.F., P.A. Christin and E.J. Edwards, 2011. The C_4 lineages of planet Earth. J. Exp. Bot., 62: 3155-3169.

Salehi, M., M. Kafi and A.R. Kiani, 2012. Salinity and water effects on growth, seed production and oil content of *Kochia scoparia*. J. Agron., 11: 1-8.

Sikolia, S., J.C. Onyango, E. Beck and J.I. Kinyamario, 2009. The distribution of C_3 and C_4 *Photosynthetic* species of the centrospermeae along an altitudinal gradient in Western Kenya. Int. J. Bot., 5: 47-57.

Spurr, A.R., 1969. A low-viscosity epoxy resin embedding medium for electron microscopy. J. Ultrastruct. Res., 26: 31-43.

Sudderth, E.A., R.M. Muhaidat, A.D. McKown, F. Kocacinar and R.F. Sage, 2007. Leaf anatomy, gas exchange and photosynthetic enzyme activity in *Flaveria kochiana*. Functional Plant Biol., 34: 118-129.

Takabayashi, A., M. Kishine, K. Asada, T. Endo and F. Sato, 1969. Differential use of two cyclic electron flows around photosystem I for driving CO_2-concentration mechanism in C_4 photosynthesis. Proc. Nat. Acad. Sci. USA., 102: 16898-16903.

Ueno, O., Y. Yoshimura and N. Sentoku, 2005. Variation in the activity of some enzymes of photorespiratory metabolism in C_4 grasses. Ann. Bot., 96: 863-869.

Vollesen, K., 2000. *Blepharis* (Acanthaceae): A Taxonomic Revision. Royal Botanic Gardens, Kew, UK., ISBN-13: 9781900347938, Pages: 354.

Vollesen, K., 2002. Three new species of *Blepharis* (Acanthaceae). Kew Bull., 57: 451-457.

Voznesenskaya, E.V. and Y.V. Gamaley, 1986. The ultrastructural characteristics of leaf types with Kranz anatomy. Botanicheskii Zhurnal, 71: 1291-1307.

Voznesenskaya, E.V., V.R. Franceschi, V.I. Pyankov and G.E. Edwards, 1999. Anatomy, chloroplast structure and compartmentation of enzymes relative to photosynthetic mechanisms in leaves and cotyledons of species in the tribe Salsoleae (Chenopodiaceae). J. Exp. Bot., 50: 1779-1795.

Voznesenskaya, E.V., V.R. Franceschi, S.D.X. Chuong and G.E. Edwards, 2006. Functional characterization of phosphoenolpyruvate carboxykinase type C_4 leaf anatomy: Immuno, cytochemical and ultrastructural analyses. Ann. Bot., 98: 77-91.

Voznesenskaya, E.V., N.K. Koteyeva, G.E. Edwards and G. Ocampo, 2010. Revealing diversity in structural and biochemical forms of C_4 photosynthesis and a C_3-C_4 intermediate in genus *Portulaca* L. (Portulacaceae). J. Exp. Bot., 61: 3647-3662.

Wingler, A., R.P. Walker, Z.H. Chen and R.C. Leegod, 1999. Phosphoenolpyruvate carboxykinase is involved in the decarboxylation of aspartate in the bundle sheath of maize. Plant Physiol., 120: 539-545.

Wintermans, J.F. and A. de Mots, 1965. Spectrophotometric characteristics of chlorophylls a and b and their pheophytins in ethanol. Biochem. Biophys. Acta, 109: 448-453.

Yoshimura, Y., F. Kubota and O. Ueno, 2004. Structural and biochemical bases of photorespiration in C_4 plants: Quantification of organelles and glycine decarboxylase. Planta, 220: 307-317.